BRIEF REPORT

Cross-Serotype Neutralization of Dengue Virus in *Aotus nancymae* Monkeys

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Previously, we observed that serum from humans immune to dengue serotype 1 (dengue-1) neutralized the American genotype of dengue serotype 2 (American-2) to a greater extent than it neutralized the Asian genotype of dengue serotype 2 (Asian-2). To determine if this activity is protective, *Aotus nancymae* monkeys were infected with dengue-1 followed by either American-2 or Asian-2. Dengue-1–infected animals produced antibody with neutralizing titers of 2656 antibodies against dengue-1, 409 against American-2, and <20 against Asian-2. Infection with American-2 did not produce detectable viremia in either dengue-1–immune or dengue-1–naive animals. These findings support the hypothesis that dengue-1 immunity might have prevented disease or altered the severity of disease in individuals sequentially infected with dengue-1 and American-2.

Dengue viruses are single-stranded, positive-sense RNA viruses that belong to the *Flavivirus* genus in the Flaviviridae family. There are 4 serotypes of dengue virus (dengue 1–4), and each serotype consists of many closely related (genotypic) viruses. Dengue viruses are transmitted primarily by *Aedes aegypti* mosquitoes, and all 4 serotypes cause human disease. It is estimated that 100 million dengue infections occur annually worldwide [1], and these can either be subclinical or cause diseases ranging from a flulike syndrome—dengue fever—to severe disease characterized by capillary leakage and thrombocytopenia—dengue hemorrhagic fever (DHF)—to hypovolemic shock—dengue shock syndrome.

Epidemiological studies have shown that the severity of disease can be dependent on the infecting serotype and on the individual's history of infection with dengue viruses [1, 2]. A primary dengue infection is usually either subclinical or causes dengue fever, and the individual will develop lifelong immunity to the infecting serotype. Heterotypic immunity is short-lived, and sequential heterotypic infections can lead to increased severity of disease, possibly by an antibody-enhancement mechanism [3–6]. Increased severity of disease is associated with sequential infections with all combinations of serotypes, but more-severe cases have been associated primarily with sequential infection with dengue serotype 1 (dengue-1) and the Asian genotype of dengue serotype 2 (Asian-2) [6–8]. In contrast, sequential infection with dengue-1 and the American genotype of dengue serotype 2 (American-2) was not associated with DHF in Iquitos, Peru [9]. Serum from Peruvians putatively infected with dengue-1 neutralized dengue-1 and American-2 to a significantly greater extent than it neutralized Asian-2 [10]. The heterologous neutralization of American-2 by dengue-1 sera may have been a contributing factor for the lack of DHF observed in individuals sequentially infected with dengue-1 followed by American-2. The present study was conducted to test the hypothesis that dengue-1 antibody–positive *Aotus nancymae* monkeys would be protected against infection with American-2.

Adult male and female *Aotus nancymae* monkeys, 750–1400 g, were maintained in a facility, at the US Naval Medical Research Center Detachment in Lima, Peru, that was approved by the Association for Assessment and Accreditation of Laboratory Animal Care. The experiments were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* [11]. Twenty-four animals negative for antibodies to dengue serotypes 1–4 were assigned to 1 of 4 groups, designated "A," "B," "C," and "D." The 12 animals in groups A and B were inoculated subcutaneously (sc) in the upper arm, by use of a 28-gauge needle, with 1×10^4 plaque-forming units (pfu) of dengue-1 strain IQT6152. On days 0–9, serum samples were collected from all animals in groups A and B and were inoculated onto C6/36 cells propagated in T-25 cm^3 flasks. Virus was identified by the indirect immunofluorescence technique by use of polyclonal antibodies specific for dengue and of monoclonal antibodies specific for dengue serotypes 1-4 [12]. On day 140, the 12 animals in groups

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A and C were inoculated sc in the upper arm, by use of a 28-gauge needle, with 1×10^4 pfu of American-2 strain IQT2124; the 12 animals in groups B and D were similarly inoculated with 1×10^4 pfu of Asian-2 strain OBS8041. On days 140–49, serum samples were collected from all animals and were inoculated onto C6/36 cells and the cells were tested for virus, as described above.

Serum samples were collected from all animals on days 0, 21, 29, 60, 88, 119, 140, 149, 162, 170, 200, and 232 and were tested, by plaque reduction neutralization test (PRNT), for antibodies to dengue-1 strain IQT6152, American-2 strain IQT2124, and Asian-2 strain OBS8041, as described elsewhere [10]. The results of the PRNT were expressed as the reciprocal of the serum dilution, determined by probit analysis, that reduced the number of plaques by 50%, compared with that in normal human serum at the same dilution. The geometric means of these reciprocals (GM-R) were compared by use of the Kruskal-Wallis test. Bonferroni correction was applied to comparisons between multiple groups. All statistical analysis was performed with SPSS software (version 10.1; SPSS).

Four groups (A–D) of 6 *Aotus nancymae* monkeys each were used in this experiment. The 12 animals in groups A and B were infected with dengue-1 and monitored, by PRNT, for antibody responses and, by cell-culture assay, for viremia. The GM-Rs of antibodies to dengue-1, as determined by PRNT, are shown in table 1. For the 12 animals in groups A and B, robust responses were seen on day 21, the first postinfection time when sampling was done. Throughout the course of the experiment, their GM-Rs of antibodies to dengue-1 remained at (or above) approximately the level seen on day 88 (average GM-R of titer on day 88, 2656). Serum with antibodies to dengue-1 neutralized American-2, and, during days 0-140, the titers peaked on day 88 (average GM-R of titer on day 88, 409; table 1). However, serum with antibodies to dengue-1 did not neutralize Asian-2, because the GM-R of the titer with regard to the latter was <20 (lowest dilution tested, 1:20) during days 0–140 (table 1). Comparison of the day-88 GM-Rs of the 2 groups revealed that they were significantly different ($P < .001$, for each pairwise comparison).

Monkeys in groups A and B were monitored for viremia for 9 days after infection with dengue-1. Viremia was detected in all animals and lasted for a mean of 4.33 days (table 2).

On day 140, animals in all 4 groups were inoculated with dengue-2. The 12 animals in groups A and C were inoculated with American-2, and the 12 animals in groups B and D were inoculated with Asian-2. Animals were monitored for viremia for 9 days after inoculation (table 2), and titers of antibodies were determined on days 149, 162, 170, 200, and 232 (table 1). Neither the dengue-naive animals in group C nor the dengue-1–immune animals in group A developed viremia after being inoculated with American-2; after being inoculated with Asian-2, all dengue-naive animals in group D developed viremia that lasted for a mean of 2.33 days, and 3 of the 6 dengue-1–immune animals in group B developed viremia that lasted for a mean of 0.83 days. The difference between the number of days of viremia in the 2 groups was significant ($P = .025$), thus demonstrating heterotypic protection when animals are sequentially infected with dengue-1 and Asian-2.

Inoculation of dengue-1–immune animals—those in groups A and B—with either American-2 or Asian-2 had no significant effect on titers of antibodies to dengue-1, but, when titers for day 140 were compared with those for day 149, levels of

Table 1. Time course and titers of antibodies to dengue virus.

Virus, animal group	Experiment interval											
	Day 0	Day 21	Day 29	Day 60	Day 88	Day 119	Day 140	Day 149	Day 162	Day 170	Day 200	Day 232
Dengue-1												
Group A	<20	2066	3563	3477	1390	3421	1938	3373	1566	4142	2390	3409
Group B	<20	10,240	9906	5846	3921	3515	2537	4416	2157	5989	2870	2874
Group C	<20	<20	<20	<20	NS	NS	<20	<20	22	20	37	22
Group D	<20	<20	<20	<20	NS	NS	<20	<20	21	29	24	37
American-2												
Group A	<20	82	101	326	434	211	156	438	2736	2633	2544	2263
Group B	<20	135	108	262	384	101	85	1316	5367	3676	3010	3258
Group C	<20	NS	NS	NS	NS	NS	<20	38	532	809	1725	2286
Group D	<20	NS	NS	NS	NS	NS	<20	78	550	1000	1076	2202
Asian-2												
Group A	<20	<20	<20	<20	<20	<20	<20	101	47	47	631	227
Group B	<20	<20	<20	<20	<20	<20	<20	341	304	488	773	380
Group C	<20	NS	NS	NS	NS	NS	<20	20	37	58	695	214
Group D	<20	NS	NS	NS	NS	NS	<20	103	233	896	1123	1073

NOTE. Data are the results of plaque reduction neutralization test and are the reciprocal of the serum dilution, determined by probit analysis, that reduced the number of plaques by 50%, compared with that in normal human serum at the same dilution. American-1, American genotype of dengue serotype 2; Asian-1, Asian genotype of dengue serotype 2; dengue-1, dengue serotype 1; NS, no sample collected.

Table 2. Viremia caused by dengue virus.

Group, animal	Days of 1st infection: dengue-1										Days of viremia	Days of 2nd infection										American-2	Asian -2	Days of viremia
	0	1	2	3	4	5	6	7	8	9		0	1	2	3	4	5	6	7	8	9			
A																								
1	−	−	+	+	+	+	+	−	−	−	5	…	…	…	…	…	…	…	…	…	…	X	…	0
2	−	−	−	+	+	+	+	+	−	−	5	…	…	…	…	…	…	…	…	…	…	X	…	0
3	−	−	+	+	+	+	+	−	−	−	5	…	…	…	…	…	…	…	…	…	…	X	…	0
4	−	−	+	+	+	+	−	+	+	−	6	…	…	…	…	…	…	…	…	…	…	X	…	0
5	−	−	+	+	−	−	−	−	+	+	4	…	…	…	…	…	…	…	…	…	…	X	…	0
6	−	−	+	−	−	−	−	−	−	−	1	…	…	…	…	…	…	…	…	…	…	X	…	0
Mean	…	…	…	…	…	…	…	…	…	…	4.33	…	…	…	…	…	…	…	…	…	…	…	…	0
B																								
1	−	−	+	+	+	−	−	−	−	−	4	−	−	−	−	−	−	−	−	−	−	−	X	0
2	−	−	+	+	+	−	−	−	−	−	3	−	−	−	−	−	−	−	−	−	−	−	X	0
3	−	−	+	+	+	−	−	−	−	−	3	−	−	−	−	−	−	−	−	−	−	−	X	0
4	−	−	+	+	+	+	+	−	−	−	5	−	−	+	+	−	−	−	−	−	−	−	X	2
5	−	−	+	+	+	+	+	+	+	−	7	−	−	+	−	−	−	−	−	−	−	−	X	1
6	−	−	+	+	−	+	+	−	−	−	4	−	−	+	+	−	−	−	−	−	−	−	X	2
Mean	…	…	…	…	…	…	…	…	…	…	4.33	…	…	…	…	…	…	…	…	…	…	…	…	0.83
C																								
1	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	X	…	0
2	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	X	…	0
3	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	X	…	0
4	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	X	…	0
5	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	X	…	0
6	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	X	…	0
Mean	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	0
D																								
1	…	…	…	…	…	…	…	…	…	…	…	…	+	+	−	−	−	−	−	−	−	−	X	2
2	…	…	…	…	…	…	…	…	…	…	…	…	+	−	−	−	−	−	−	−	−	−	X	1
3	…	…	…	…	…	…	…	…	…	…	…	…	+	−	−	+	+	−	−	−	−	−	X	3
4	…	…	…	…	…	…	…	…	…	…	…	…	+	−	−	−	−	+	+	−	−	−	X	3
5	…	…	…	…	…	…	…	…	…	…	…	…	+	−	+	+	−	−	−	−	−	−	X	3
6	…	…	…	…	…	…	…	…	…	…	…	…	+	+	−	−	−	−	−	−	−	−	X	2
Mean	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	2.33

NOTE. The time course of infections are given in days. X, inoculated with virus; +, virus isolated; −, no virus isolated.

antibodies to dengue-1 had increased (group A, $P = .522$; group B, $P = .055$; table 1). A similar increase ($P = .078$) in titers of antibodies to American-2 was seen in animals in group A (table 1), and a significant increase in titers of antibodies to American-2 was seen in animals in group B ($P = .010$; table 1). Animals in group A and animals in group B showed a significant increase in their titers of antibodies to Asian-2 (group A, $P = .002$; group B, $P = .002$; table 1). Dengue-naive animals that were infected with either American-2—those in group C—or Asian-2—those in group D—developed antibodies to dengue-2 (table 1).

Our results indicate that serum from dengue-1–infected monkeys neutralizes dengue-1 and American-2 to a significantly greater extent than it neutralizes Asian-2. These results support our findings published elsewhere—that serum from dengue-1–infected humans neutralizes American-2 to a significantly greater extent than it neutralizes Asian-2 [10]. We hypothesize that this heterotypic neutralization protected against development of DHF in Peruvians who were first infected with dengue-1 and were subsequently infected with American-2. But, because infection with American-2 did not produce viremia in the *Aotus nancymae* monkeys, we were not able to determine if the heterotypic neutralization was protective. It must be noted, however, that if the heterotypic neutralization was protective, it did not confer sterile immunity, because all animals infected with American-2 produced antibodies to dengue-2, suggesting that the virus replicated in the animals. It is likely that this heterotypic neutralization contributed to protection against DHF in dengue-1–immune Peruvians who were subsequently infected with American-2 during an epidemic in 1995 [9].

The neutralization of American-2 by serum with antibodies to dengue-1 was not due to the short-term, cross-serotype responses that are often observed immediately after a dengue virus infection, because serum with antibodies to dengue-1 that was collected on days 21–140 did not neutralize Asian-2 at the lowest dilution tested. Cross-serotype protection was, however, evident in the dengue-1–immune animals that were sequentially infected with Asian-2, because the duration of viremia was reduced from a mean of 2.33 days in dengue-1–naive animals to a mean of 0.83 days in dengue-1–immune animals. It is not clear if the cross-serotype protection resulted from the minimal level of neutralization of Asian-2 observed in the animals in group B or from cellular activities, which were not measured in the present study.

Viruses of the American-2 type have been shown to be less pathogenic to humans than are viruses of the Asian-2 type [9, 13]. Studies focused on identifying the mechanism of the disproportionate pathogenesis have identified differences in the replication competency of the viruses in field isolate mosquitoes and human cells [14–16]: in both hosts, the level of replication by American-2 lagged behind that by Asian-2. Possibly, this difference in competency to replicate is responsible for both the lack of detectable viremia and the lag in production of antibodies in American-2–infected animals, compared with Asian-2–infected animals. Possibly, the competency of dengue viruses to replicate in *Aotus nancymae* monkeys may be a surrogate marker for the virulence of the viruses in humans.

A hypothesis that is consistent with the observed heterotypic neutralization present in sequential infection with dengue-1 and American-2 is that an epitope (or several epitopes) common to dengue-1 and American-2 is absent from Asian-2. Because the envelope protein is the major determinant of viral antigenicity, it is likely that this protein contains the heterotypic virus-neutralizing epitope(s) of dengue-1 and American-2. Comparison of the envelope proteins of dengue-1, American-2, and Asian-2 reveals that 4 amino acids consistently differ between American-2 and Asian-2 and that 2 of them are conserved in dengue-1. Current efforts are being directed toward the identification of the heterotypic virus-neutralizing epitope(s) of dengue-1 and American-2.

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